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**13. ABSTRACT (Maximum 200 Words)**

Considerable progress has been made toward tasks aimed at reconstitution, mapping and determining the specificity of the SNAIL-SNAG domain/SNAP interaction *in vitro* and *in vivo*. We have obtained epitope-tagged full length cDNA clones of the SNAG domain zinc finger protein, SNAIL, and the SNAPs, Ajuba and LIMD1 and verified stable protein expression in mammalian cells. We have demonstrated that Ajuba can interact with SNAIL by co-immunoprecipitation and have shown that wild type but not SNAG-domain-deleted SNAIL can augment SNAIL-mediated repression of transiently transfected E-Cadherin promoter Luciferase reporter plasmids. Immunocytochemical analysis has shown that in the absence of cotransfected SNAG domain proteins, both Ajuba and LIMD1 exhibit predominantly cytoplasmic subcellular localization: however, Ajuba deleted for nuclear exclusion signals localizes in both the cytoplasm and nucleus. We have generated a panel of HEK293 stable cell lines expressing Ajuba and the LIMD1 and examined differences in the biochemical complexes associated with Ajuba and LIMD1 in cytoplasmic compared to nuclear fractions. We have generated and characterized extremely useful polyclonal antibodies capable of detecting and discriminating between Ajuba and LIMD1. Our current efforts are aimed at definition of potential dominant negative SNAG/SNAP interaction surfaces towards our goal of reactivating E-Cadherin to control the metastatic phenotype.

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**Introduction:** This research project is based on the reported finding of an inverse expression correlation in a spectrum of human tumors: an elevated level of SNAIL, a SNAG repression domain-containing zinc finger protein, is correlated with the loss of E-Cadherin expression. Concurrently, it has been demonstrated that SNAIL acts as a potent transcriptional repressor of E-Cadherin expression via direct binding to E-box elements (GCAGGTG) in the E-Cadherin promoter [1], and this repression is abolished by mutation of the SNAG domain. It is known that the E-Cadherin glycoprotein regulates the cellular adhesive properties required to establish and maintain the tissue architecture of many epithelial cell types, including normal breast epithelia. Loss of E-Cadherin expression in breast carcinoma is implicated in the loss of tumor cell adhesion and the acquisition of the metastatic phenotype. This progression of benign tumors to invasive carcinoma is the single most important obstacle that must be confronted in order to eliminate the morbidity and mortality from this disease. Our proposed studies will apply strategies for reactivating E-Cadherin expression, with the long-term goal of halting breast tumor progression and metastatic spread. We have specifically proposed that targeting the SNAG repression function of SNAIL should lead to reactivation of E-Cadherin and have made significant progress in establishing reagents for testing this hypothesis in model systems.

**Body:** Our preliminary studies were focused on the following characterization of the SNAG domain: 1. the SNAG of Gfi-1domain was demonstrated using reporter assays to be a dominant, transferable, DNA binding-dependent repression module [2, 3], 2. targeted mutagenesis was applied to define the amino acid residues critical for SNAG-mediated repression, 3. a yeast two-hybrid screen was employed to isolate two SNAG domain Interacting Proteins (SNAPS). The first, SNAP13, had been previously cloned as the LIM domain protein [4, 5] designated LIMD1 [6], and the second, SNAP20, was also an independently isolated LIM domain protein designated, AJUBA [7,8]. 4. Our characterization has determined that in the presence of a wild-type SNAG protein, which is capable of interaction with AJUBA, AJUBA displays a distinctly nuclear subcellular localization; however, in the absence of a SNAG protein or in the presence of a mutant SNAG protein, AJUBA is cytoplasmic. Our finding that mutations in the SNAG domain that abolish repression, also prevent nuclear localization of AJUBA suggest that we have discovered a novel co-repressor that mediates SNAG domain repression via nuclear-cytoplasmic shuttling.

These basic characterizations of the SNAG domain have led us to our proposal to examine the SNAIL-SNAG protein and its regulation of E-Cadherin [1]. Because of its significant role in metastatic breast cancer, our goal is to extend these preliminary studies of model SNAG:SNAP interactions to the context of E-Cadherin, the target gene of the SNAG zinc finger protein SNAIL. The proposed strategies are aimed at disrupting the interactions between the SNAG domain of SNAIL with its endogenous SNAP in order to abolish SNAIL-mediated repression of the E-Cadherin gene and lead to a reversal of the metastatic phenotype. The Specific Aims defined in our original proposal included the following: (1) Reconstitute, map and determine the specificity of the SNAIL-SNAG domain-SNAP interaction *in vitro* and in vivo. (2) Identify dominant negative and peptide-based inhibitors of the SNAG-SNAP interaction and use them as tools to manipulate SNAIL-mediated repression in vivo. (3) Define the set of genes that are under control of the SNAIL in breast cancer cells using cDNA microarray analysis.

We are enthusiastic about our accomplishments toward satisfying tasks associated with Specific Aim 1. The major goal of Aim 1 was to: Reconstitute, map and determine the specificity of the SNAIL-SNAG domain/SNAP interaction *in vitro* and *in vivo*. We have obtained full-length cDNAs for the SNAP proteins AJUBA and LIMD1 and subcloned these as fusion cDNAs with a 6xHIS affinity tag and a FLAG epitope tag at the NH2-terminus to a

pcDNA3 mammalian expression vector. These SNAP expression constructs are depicted in Figure 1A. For AJUBA, an expression construct containing a specific deletion of the AJUBA nuclear exclusion signal (NES) was also constructed. The autoradiogram in Figure 1B validates that these constructs permit stable protein expression in COS-1 cells as demonstrated after transient transfection, metabolic $^{35}S$ labeling, and immunoprecipitation with the M2 anti-FLAG antibody, or with the 9E10 monoclonal antibody against the myc epitope-tagged Ajuba protein expressed from the pCS2 plasmid (obtained from our collaborator, Dr. Longmore). When transiently overexpressed in Hep2 cells and detected by immunofluorescence microscopy using the anti-FLAG M2 antibody (Figure 1C) it can be readily observed for the FLAG-LIMD1 protein, and to a lesser degree for the His-FLAG-Ajuba protein, that both proteins containing the NES domain exhibit a predominantly cytoplasmic localization, however the Ajuba protein (-NES) lacking the nuclear exclusion signals is abundantly distributed in both cytoplasmic and nuclear subcellular locations.

To aid in studies localizing these endogenous proteins, we have developed specific antibodies to both Ajuba and LIMD1. We have subcloned unique portions of the mouse Ajuba cDNA (encoding amino acids 1-216) and the mouse LIMD1cDNA (encoding amino acids 1-158) into the pQE30 bacterial expression vector as fusions to the 6xHIS affinity tag. These recombinant proteins (depicted in Figure 1D), were expressed in the E. coli BL21(DE3) strain after IPTG induction, were then purified from crude cell lysates to greater than 95% using Ni-NTA affinity chromatography and were resolved by SDS-PAGE. These purified Ajuba and LIMD1 antigens were then injected into rabbits to generate polyclonal antiserum. Two rabbits were used for each antigen and the production bleeds were obtained at different time intervals subsequent to booster injections. The rabbit sera were screened for reactivity by immunoprecipitation of $^{35}S$ methionine labeled Ajuba and LIMD1 protein antigens produced after translation *in vitro*. Depicted in Figure 2A are the autoradiograms showing that every bleed of LIMD1-immunized rabbits #3201 and #3149 produced highly reactive sera for the LIMD1 antigen. The rabbits injected with the Ajuba protein, #3215 and #3198 produced sera weakly reactive against the Ajuba antigen. Figure 2B demonstrates that the LIMD1 serum is specific for LIMD1 as it does not react with the Ajuba antigen. These sera were further screened by immunoprecipitation against the full length Ajuba and LIMD1 proteins produced and radiolabeled after translation *in vitro*. Figure 1E depicts the autoradiogram demonstrating that the polyclonal sera are capable of detecting the full length Ajuba (~58 kDa) and the LIMD1 (~71 kDa) proteins. Hence we have successfully generated antisera that should be useful in detecting and discriminating between these proteins when expressed endogenously.

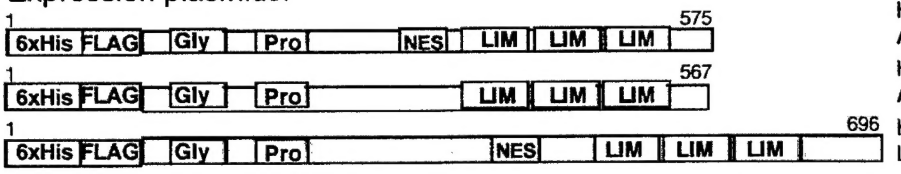
We have also obtained a full length SNAIL cDNA in order to examine interactions between SNAIL and SNAP proteins. To determine if AJUBA can recognize the SNAG domain in the context of a full length SNAG-zinc finger protein, we co-transfected myc-AJUBA with a vector that expresses full length SNAIL protein containing a FLAG epitope tag at the COOH terminus (Fig 3A). We also constructed a SNAIL vector that lacks the SNAG domain (delta-SNAIL-flag). Each protein was abundantly expressed as evidenced by western blot analyses of the cell extracts. Immunoprecipitation using the FLAG antibody followed by western blotting with the myc antibody showed abundant AJUBA in the SNAIL transfected cells (Fig 3A). The amount of AJUBA recovered in the I.P. was greatly reduced (but not absent) in the delta-SNAIL-flag transfected cells. Thus, full length AJUBA can bind to full length SNAIL and the SNAG domain augments this interaction. To determine if AJUBA could function as a co-repressor for the full length SNAG domain zinc-finger protein, we co-transfected SNAIL-FLAG with the full length AJUBA. Each dish also received a luciferase reporter plasmid that contains the proximal E-Cadherin promoter. This construct contains at least three SNAIL binding sites in the promoter

region that bind with high affinity to the SNAIL protein. [34]. Hela cells support SNAIL mediated repression when transfected alone (Figure 3B left panel). However, the silencing activity does not respond to increasing concentrations of SNAIL plasmid, implying that a limiting factor is required. When, a fixed amount of SNAIL is transfected with increasing amounts of AJUBA, a very strong repression activity is observed [1] (Figure 3B middle panel). This augmentation of repression activity was not observed when the delta-SNAIL is used (data not shown). Thus AJUBA can function as a co-repressor for full length SNAIL, in a SNAG domain dependent manner, on a physiologically relevant target gene.

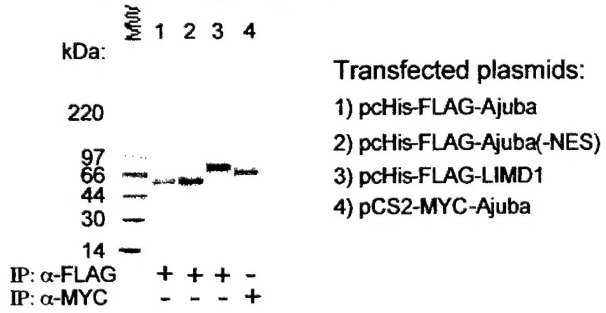
We have furthermore generated a panel of HEK293 cell lines that stably express the Ajuba protein, the Ajuba (-NES) protein, and the LIMD1 protein. Our immunoblot analysis of the stable cell lines using the anti-FLAG M2 antibody is shown in Figure 4A. These clones will serve as a model system for the isolation of biochemical complexes of SNAP associated proteins. Both nuclear and cytoplasmic fractions were subjected to purification and the progress followed using the anti-FLAG antibody. The silver-stained gel in Figure 4B reveals that although there is a set of proteins uniquely associated with LIMD1, but not Ajuba in the cytoplasm, that in the nuclear fraction both Ajuba (-NES) and LIMD1 appear to share some common associated proteins. These studies will identify both the nuclear and cytoplasmic components of a novel signaling system involving GFI1 and AJUBA. These purifications will be repeated using the antibodies to Ajuba and LIMD1. Stochiometrically associated polypeptides will be subjected to MALDI-mass spectrometry for identification. Identification of these associated polypeptides will very be informative with respect to the role in both SNAG-mediated transcriptional repression and in the biological function of GFI1 and AJUBA.

Our current efforts are directed at finishing tasks within Specific aim 1 by creating a panel of deletion and point mutations based on domain boundaries and subcloning these constructs to bacterial and mammalian expression vectors (constitutive and inducible) that incorporate epitope tags. Our initial mutagenesis efforts will target the SNAP LIM domains. We will continue to perform *in vitro* interaction assays using a combination of GST chromatography, and co-immunoprecipitation using transient co-transfection of tagged SNAP and SNAIL expression constructs and co-immunoprecipitation of cell extracts (as above). We will also engineer a set of point mutations focusing on the potential protein-protein interaction surfaces in each SNAP. Finally, to determine the specificity of the interaction we will utilize the other LIM domain family members of which SNAPs are a member HIC5 [9, 10], TRIP6 [11], Zyxin [12] and LPP [13]. We will utilize epitope tag versions and we will determine whether these proteins can interact directly with the SNAIL-SNAG domain via the assays described above. The outcome of these studies will enable us to identify dominant negative and peptide-based inhibitors of the SNAG-SNAP interaction and use them as tools to manipulate SNAIL-mediated repression *in vivo* as described in Specific Aim 2. The goal of this aim is to utilize truncated versions of the SNAIL and SNAP proteins as dominant negatives in vivo in order to manipulate E-Cadherin expression in the MDA-MB 435 cell lines that are highly malignant and have low E-Cadherin expression to evaluate the metastases potential in SCID mice based models.

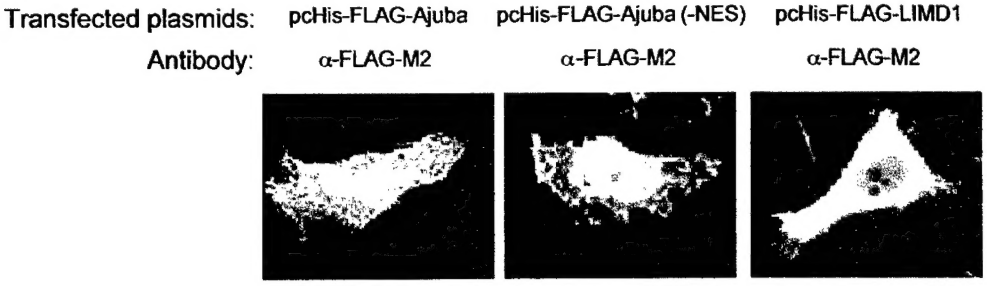
**A.** Expression plasmids:




**B.** COS-1 Transfection and Immunoprecipitation




**C.** Immunolocalization of His-FLAG-Ajuba, His-FLAG-Ajuba (-NES) and His-FLAG-LIMD1 proteins




**D.** Ajuba and LIMD1 antigens


**E.** Characterization of Ajuba and LIMD1 antibodies

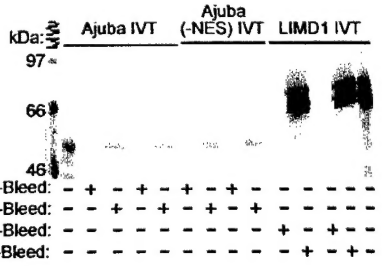



**Figure 1: A**. The mammalian expression plasmids were constructed by sub-cloning the cDNAs encoding His-FLAG-Ajuba, His-FLAG-Ajuba(-NES) and His-FLAG-LIMD1 genes into pcDNA3 vector. The pcHis-FLAG-Ajuba(-NES) construct lacks amino acids 289-297 of Ajuba, which harbors a functional leucine-rich nuclear export signal. **B**. Indicated expression plasmids were transfected into COS-1 cells, the metabolically labeled extracts were immunoprecipitated with α-FLAG antibody and analyzed. **C**. HEp2 cells that were transiently transfected with the expression plasmids, immunostaining was carried out with α-FLAG-M2 antibody and single cell images are presented. **D**. BL21(DE3) recombinants of pQE30-Ajuba or pQE30-LIMD1 were induced with IPTG and the crude cell lysates were bound to $Ni^{2+}$-NTA beads. After washing the beads, the bound proteins were serially eluted using buffer containing 400 mM imidazole and portions were analyzed on 12% SDS-PAGE. **E**. The $^{35}S$-labeled Ajuba, Ajuba (-NES), and LIMD1 IVT proteins were immunoprecipitated with either pre- or test-bleeds and analyzed by fluorography. Note that the Ajuba antiserum detects both Ajuba and Ajuba (-NES) proteins.

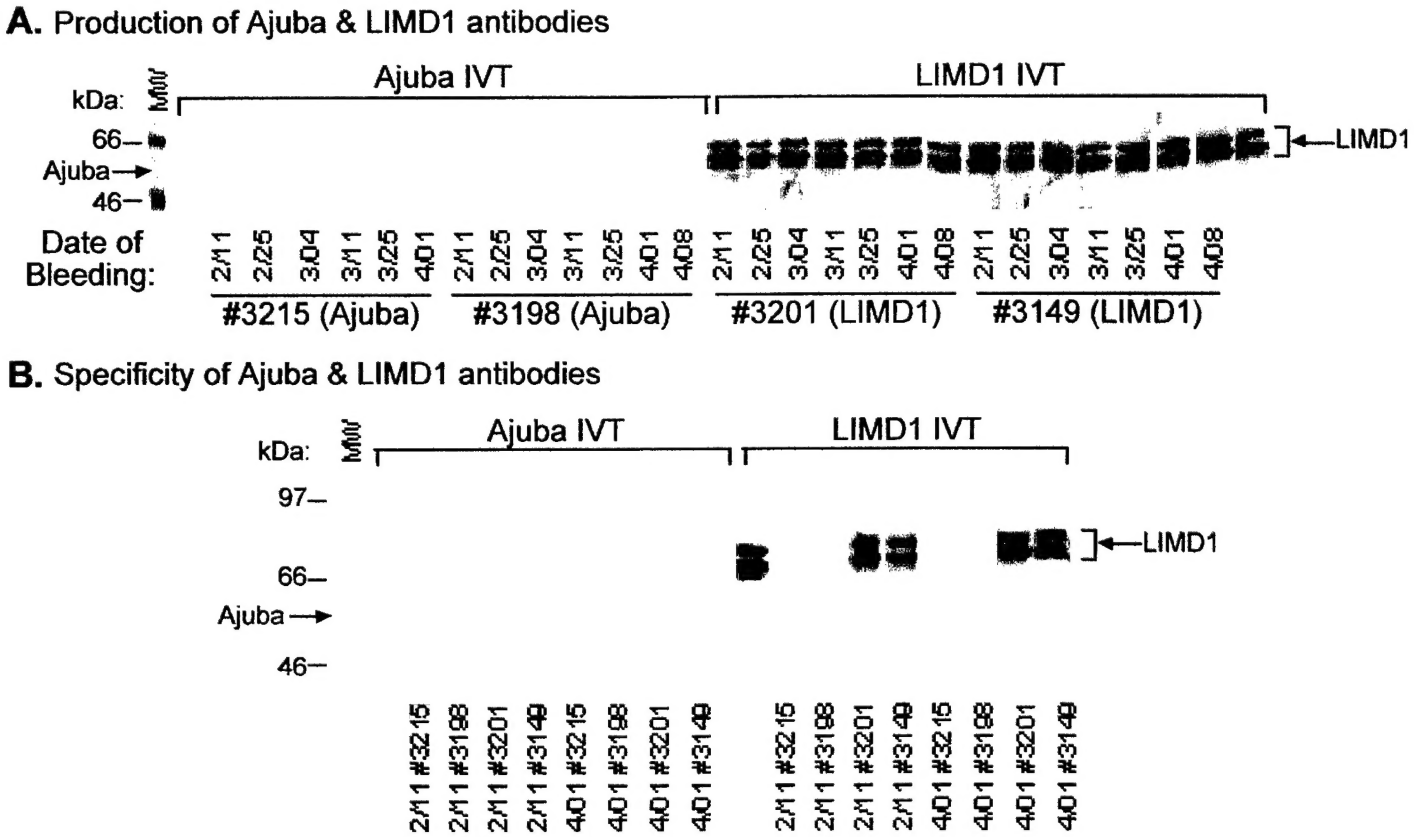



Figure 2. A. Production and Characterization of Ajuba and LIMD1 antibodies. Purified recombinant Ajuba and LIMD1 antigens were injected into rabbits in order to generate polyclonal antibodies. Two rabbits were used foreach antigen. The production bleeds were obtained at different time intervals subsequentto booster injections and tested using 35-S-methionine labeled Ajuba and LIMD1 in vitro translated proteins (IVT) as antigens by immunoprecipitation. The immunoprecipitated proteins were electrophoresed on 10% SDS-PAGE along with 14-C labeled protein molecular weight markers and Ajuba and LIMD1 IVT proteins as inputs. B. Determination of specificity of Ajuba and LIMD1 antibodies. The first and sixth production bleeds of Ajuba and LIMD1 antisera were tested for specificity using both the antigens. The rabbits #3215 and 3198 were immunized with Ajuba antigen whereas the rabbits #3201 and 3149 were immunized with LIMD1 antigen.

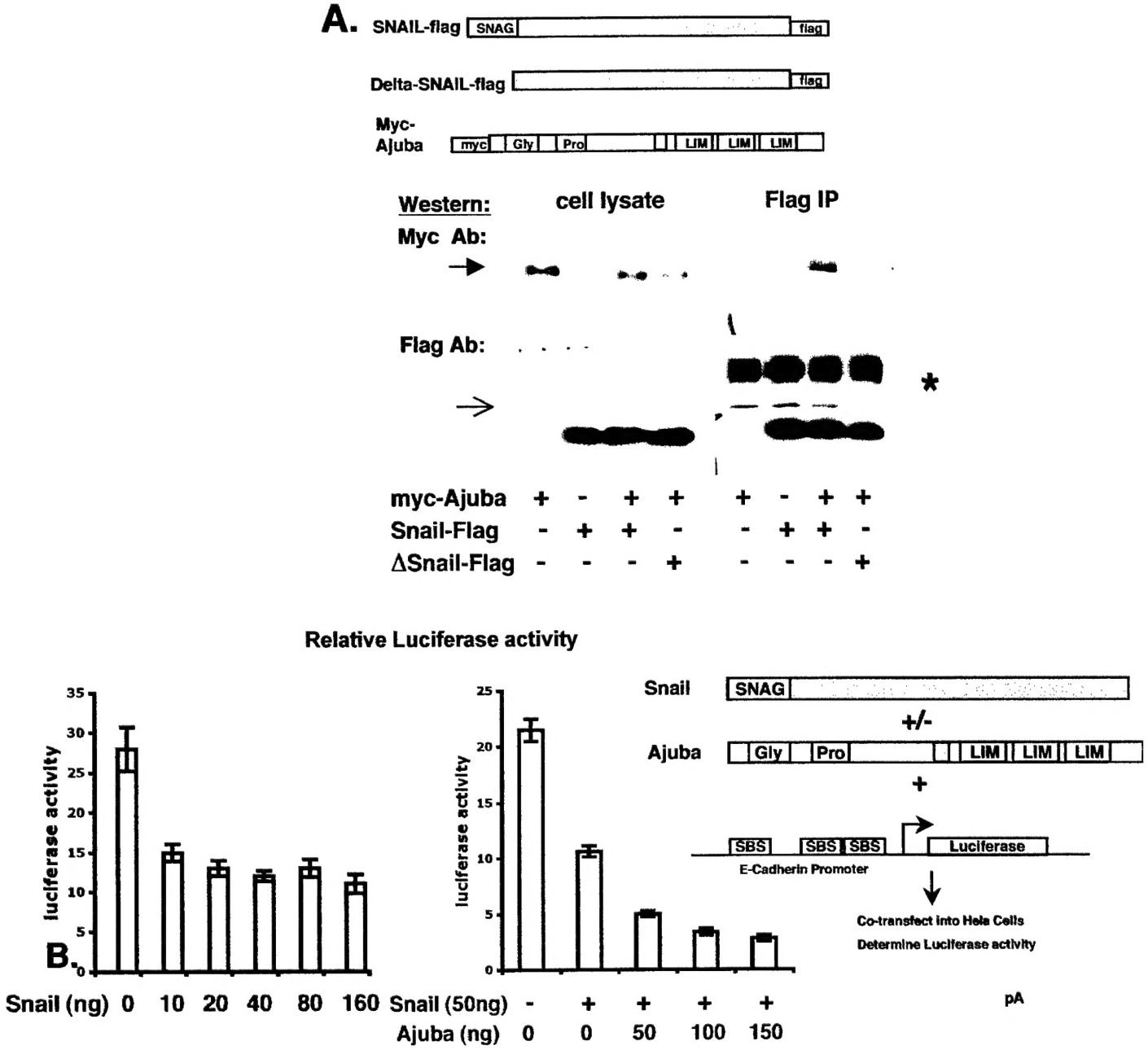
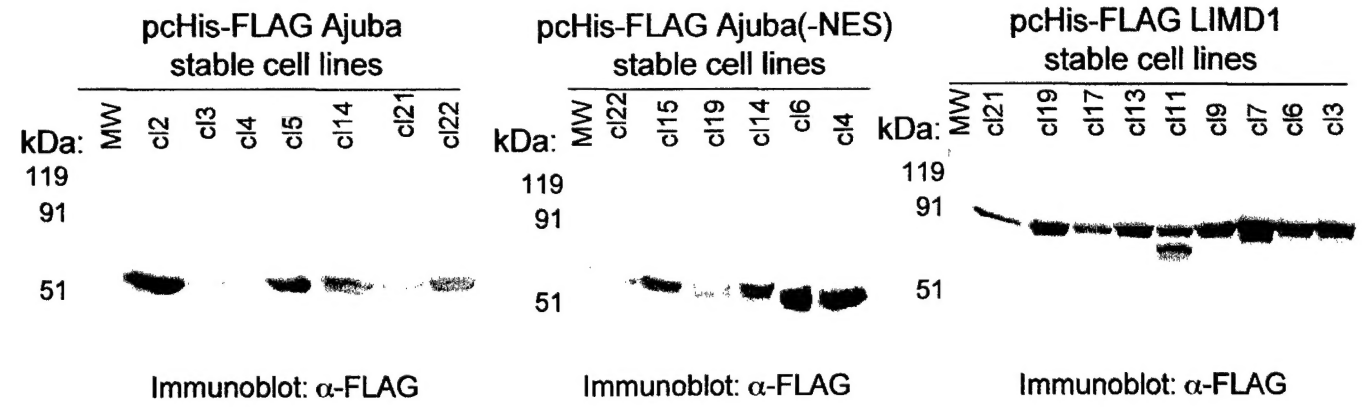



**Figure 3.** **(A)** Full-length myc-AJUBA (solid arrow) was cotransfected with either full-length SNAIL-Flag (open arrow) or a similar construct that deletes the NH2-terminal SNAG domain (delta-SNAIL-Flag). Cell lysates were either probed directly with Myc or Flag antibodies in a western blot (left panel) or were first immunoprecipitated with Flag antibodies followed by western blotting with Myc antibodies. The asterisk indicates the IgG bands. **(B)** Full length Snail was cotransfected with the E-cadherin promoter luciferase plasmid which contains natural Snail Binding Sites (SBS) (Left Panel). A fixed amount of Snail was cotransfected with increasing amounts of full length AJUBA (middle panel).

**A.** Stable clones expressing His-FLAG-Ajuba, His-FLAG-Ajuba (-NES) & His-FLAG-LIMD1 proteins




**B.**

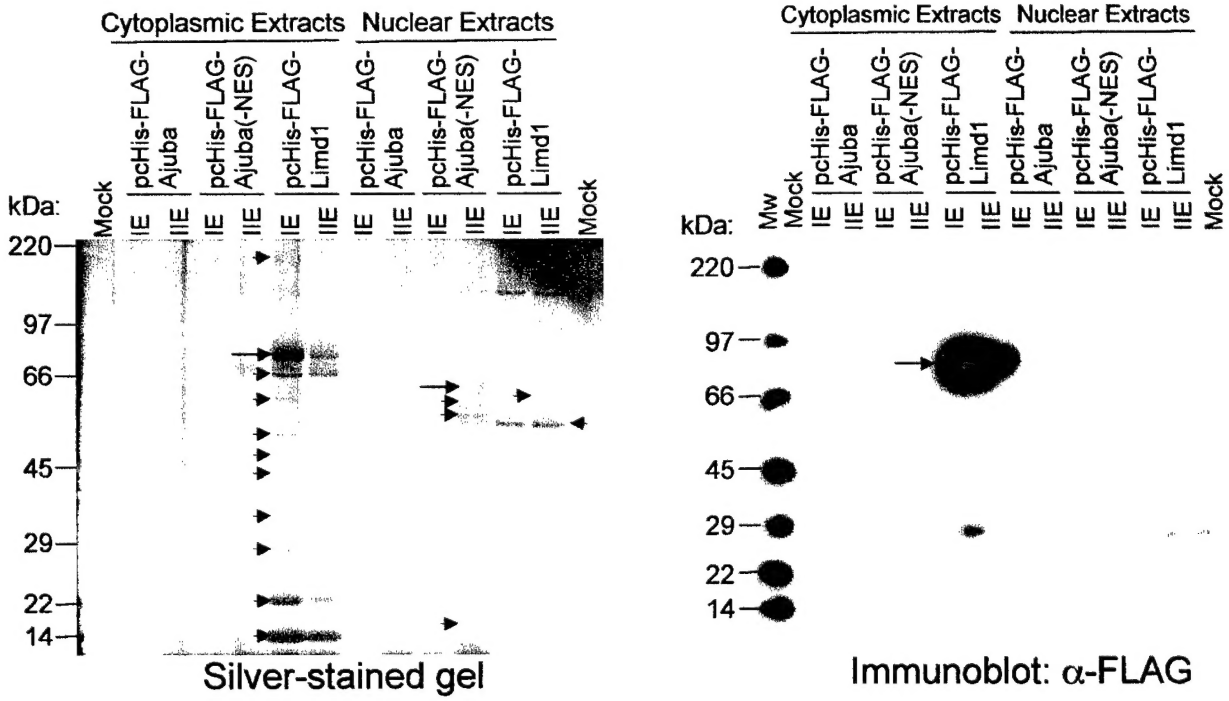



**Figure 4**: **A.** HEK 293 cells were transfected with the pcHis-FLAG-Ajuba, pcHis-FLAG-Ajuba (-NES) and pcHis-FLAG-LIMD1expression plasmids and selected for stable integrands using G418. Cell lysates were prepared from twenty-four single cell clones and analyzed by immunoblotting with α-FLAG antibody as primary and alkaline phosphatase labeled α-mouse IgG as secondary antibodies. Presence of immunoreactivity was tested by developing the color using BCIP and NBTsubstrates. Each panel illustrates mainly the good expressors. **B.** Nuclear and cytoplasmic fractions obtained from the transfected cells were incubated with the a-FLAG affinity resin. After washings, the bound proteins were eluted using FLAG peptide and electrophoresed on two 4-12% Nu-PAGE gels. One gel was processed for silver staining while the other gel was immunoblotted wit h α-FLAG antibody. Mock represents the extracts prepared f rom cells transfected with pCMV2-FLAG vector. IE and IIE stand for first and second elutions respectively. The filled block arrow represents the His-FLAG-LIMD1 protein and the open block arrow depicts the His-FLAG-Ajuba (-NES) protein. The arrowheads represent the associated polypeptides that are unique.

**Key Research Accomplishments:**

- We have obtained epitope-tagged full length cDNA clones of the SNAG domain zinc finger protein, SNAIL, and the SNAPs, Ajuba and LIMD1 and verified stable protein expression in mammalian cells.

- We have demonstrated that Ajuba can interact with SNAIL by co-immunoprecipitation and have shown that wild type but not SNAG-domain-deleted SNAIL can augment SNAIL-mediated repression of transiently transfected E-Cadherin promoter Luciferase reporter plasmids.

- We have generated a panel of HEK293 stable cell lines expressing Ajuba and the LIMD1 proteins and examined differences in the biochemical complexes associated with Ajuba and LIMD1 in cytoplasmic compared to nuclear fractions.

- We have generated and characterized extremely useful polyclonal antibodies capable of detecting and discriminating between Ajuba and LIMD1.

**Key Reportable Outcomes:**

**Manuscripts:**

1. Kasirajan Ayyanathan, Rakesh Goyal, Greg Longmore, and Frank J. Rauscher III. Functional analysis of the SNAG repression domain from the GFI-1 proto-oncogene: Identification of a novel LIM domain protein that functions in the repression pathway (Manuscript in preparation for PNAS).

**Presentations:** **None**

**Cell Lines developed:**

HEK293 stable cell lines expressing Ajuba and the LIMD1 proteins.

**Antibodies developed:**

Rabbit sera #3201 and #3149 highly reactive for the LIMD1 antigen (amino acids 1-158)

Rabbit sera #3215 and #3198 reactive for the Ajuba antigen (amino acids 1-216)

**Funding applied for based on work supported by this award:**

NIH RO1 CA 095561 Functions of the SNAG Repression domain in oncogenesis

**Research opportunities based on training provided by this approval:**

Hongzhuang Peng Ph.D.

**Conclusions:** We have specifically proposed that targeting the SNAG repression function of SNAIL should lead to reactivation of E-Cadherin and have made significant progress in establishing reagents for testing this hypothesis in model systems. We have obtained epitope-tagged full length cDNA clones of the SNAG domain zinc finger protein, SNAIL, and the SNAPs, Ajuba and LIMD1 and verified stable protein expression in mammalian cells. We have demonstrated that Ajuba can interact with SNAIL by co-immunoprecipitation and have shown that wild type but not SNAG-domain-deleted SNAIL can augment SNAIL-mediated repression of transiently transfected E-Cadherin promoter Luciferase reporter plasmids. We have generated a panel of HEK293 stable cell lines expressing Ajuba and the LIMD1 proteins and examined differences in the biochemical complexes associated with Ajuba and LIMD1 in cytoplasmic compared to nuclear fractions. We have generated and characterized extremely useful polyclonal antibodies capable of detecting and discriminating between Ajuba and LIMD1.

Our current efforts are directed at creating a panel of deletion and point mutations of Ajuba and LIMD1 proteins to map the protein-protein interaction surfaces with the SNAIL SNAG domain, and test the interaction specificity against other SNAPs (HIC5 [9, 10], TRIP6 [11], Zyxin [12] and LPP [13]). The goal of these studies is to identify dominant negative and peptide-based inhibitors of the SNAG-SNAP interaction. These tools will be used to manipulate SNAIL-mediated repression *in vivo* and to test whether reactivation of E-Cadherin expression will significantly inhibit the metastatic potential of MDA-MB 435 cell lines in SCID mice based models.

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